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HERITABILITY OF RESISTANCE TO WITCHES' BROOM IN *THEOBROMA CACAO*

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EXECUTIVE SUMMARY

During the project the automated inoculation/incubation system was perfected so that all variables are controlled that influence infection of cacao (*Theobroma cacao*) by the witches' broom pathogen (*Crinipellis perniciosa*). Use of the system to inoculate thousands of seedlings from 24 open pollinated clones discriminated more than 300 resistant/tolerant individual seedlings, and each seedling given a GNV clonal number. More than 200 GNV clones were propagated vegtetatively, the resulting plants were inoculated using the automated ioculation/incubation system, and 34 GNV clones were selected for their apparent rresistance/tolerance to witches' broom. Selected clone were deposited in the Cocoa Genebank, CRU, UWI, Trinidad.

Storage of basicidiospores of *C. perniciosa* in liquid nitrogen without loss of infectivity for as long as 7 years was the breakthrough knowledge that now makes basidiospores available as needed.

GNV clones and basidiosportes of *Crinipellis perniciosa* that are in liquid nitrogen storage will be transferred to the ACRI Cocoa Biotechnology, Pennsylvania State University, University Park, PA.

The automated inoculation/incubation system and the technology to use it were installed at the INIAP, Estación Experimental Tropical Pichilingue, Quevedo, Ecuador where excellent advances have been made. For the first time ever, it is now possible to evluated cacao germplasm effectively and efficiently for resistance/tolerance to witches' broom by using the technology and ancillary equipment that is the total automated inoculation/incubation system at Pichilingue. Restoration of the "naciónal" chacteristics into the cacao industry of Ecuador is a country-wide goal, and a contest is in progress in Ecuador to find the best "naciónal" cacao tree in the country. Candidate trees are screened with the automated inoculation/incubation system. This contest would not have been initiated before before this project because there were no effective and efficient procedures available. A newly assigned cacao breeder at Pichilingue makes the future for cacao much brighter than it has ever been, because germplasm identified by the automated inoculation/incubation system will be used to improve cacao planting materials for grower use. This was not possible previously.

The use of RAPDs to detect markers that might be indicative of resistance/tolerance to witches' broom in cacao has shown considerable promise. The technological procedures for the use of RAPDs with cacao have been established, and their application to witches' broom rsesistance/tolerance markers appears to be very near.

(4) RESEARCH OBJECTIVES

The witches' broom disease of cacao (*Theobroma cacao* L.) occurs in all cacao regions of South America, and in certain Caribbean islands. This devastating disease is not present in Central America, although there was an outbreak of the disease in Panamá in the area on the South America side of the canal.
The pathogen was isolated and named *Marasmius perniciosus* by Gerhold Stahel in 1915, he had worked previously with the disease and pathogen since the 1890's. The pathogen was reclassified in 1942 and placed in the genus *Crinipellis* with the presently accepted binomial, *Crinipellis perniciosa* (Stahel) Singer.

Witches' broom destroyed production of cocoa beans in many plantations in all countries where it occurs. As a consequence, some plantations have been abandoned, and disease pressure and disease incidence in neighboring active plantations has increased significantly. A recently completed study of the comparative epidemiology of witches' broom in several countries in South America (Rudgard, et al, 1993) demonstrated that phytosanitation (the removal from the tree of all diseased plant parts) can reduce losses caused by the disease. Evaluations of the efficacy of fungicidal chemicals against witches' broom has been reported, and the applications of copper-containing formulations have provided a standard for evaluation of new candidate materials. Whereas reduced disease incidence may occur following phytosanitation, or the applications of efficacious fungicides, the significant drain on the limited resources of many cacao growers precludes the use of either or both practices as viable choices for disease management. The only other effective practices for disease management that seemingly offers relief from present-day losses is the use of resistant/tolerant plant materials.

Diverse cacao germplasm has been collected in the accepted center of origin of cacao, the Amazon Basin region of South America, and thousands of accessions reside in several germplasm collections located in the Americas. Very few of these accessions, or clones, have been evaluated for any trait, especially for their resistance/tolerance to witches' broom. Clones free from symptoms of witches' broom (such as SCA 6, SCA 12, Pound 7, etc.) selected in Trinidad in the late 1930's soon became the base for resistance breeding to combat witches' broom.. Unfortunately, the suspected resistance of these and other clones, as well as their progenies, failed and the resulting plant materials from breeding efforts developed witches' broom at almost all other locations, including Ecuador.

Breeding cacao for resistance/tolerance to witches' broom has been ineffective for several reasons. The inability to manipulate the pathogen to produce basidiospores (the only ineffective propagule of the pathogen) when needed prevented effective evaluation of germplasm. Germplasm used in breeding efforts to develop resistant/tolerant planting materials of cacao had not been evaluated, or had been subjected to ineffective evaluations for resistance/tolerance to the disease (Evans, 1978; Holliday, 1955). For these, and other reasons, there are no plant materials available for grower use that possess adequate resistance/tolerance to witches' broom.

Today effective management of cacao in the presence of the witches' broom disease by any methods that might be employed is doomed because the primary ingredient for success is not available, resistant/tolerant planting materials.

Therefore, management must rely on labor intense and very costly methods, phytosanitation or fungicide applications. When returns to growers are low, as they are today (1994) and for the past 5 or more years, effective management practices that consume scarce resources are just not used. As a result, witches' broom runs rampant through plantations, as it is doing in the Los Rios region of Ecuador and in Bahia, Brazil where almost all producing plantations contain very susceptible germplasm. Any management effort by conscientious growers will ultimately be ineffective because inoculum of the pathogen will increase drastically in neighboring abandoned plantations where nothing is done because of scarce resources. The solution is resistant/tolerant planting materials to provide a low cost management tool to increase the probability for successful management of cacao in the presence of witches' broom. Even with resistant/tolerant planting materials, other management tactics will be essential for effective disease management when background inoculum abounds.

The desperate need for effective host resistance/tolerance to witches' broom to combat the ravages of the disease stimulated this project. The automated system and many ancillary items installed at the Estación Experimental Tropical "Pichilingue", INIAP, Quevedo, Ecuador (hereafter referred to as Pichilingue) provides for the first time at Pichilingue an effective and efficient procedure to screen germplasm of cacao for resistance/tolerance to the witches' broom disease. Restoration of the "naciónal" flavor in the cacao industry of Ecuador has evolved into a national objective. The personnel at the Pichilingue, as well as others concerned about cacao in Ecuador can address this objective in an orderly manner with the expectation that resistance/tolerance coupled with the "nacional" flavor will be detected in the diverse germplasm in the country. It is most interesting that a national contest is in progress to find the "BEST" cacao tree in all of Ecuador. A prize of 3,000,000 Sucres is offered for the wining selection. The contest is sponsored by Latinreco S.A., a chocolate-producing company within the Nestle group. Candidate trees are screened at Pichilingue using the automated inoculation/incubation system installed at the location as part of this project.

Development of the inoculation/incubation system to evaluate responses of cacao to *Crinipellis perniciosa* was supported by the American Cocoa Research Institute of the Chocolate Manufacturer's Association of the United States, McLean, VA. Other agencies that contributed to the project by providing salaries for research personnel and their associates/assistants, offices, laboratories, greenhouses, field plots, and other resources were: the Plant Pathology Department, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL; the Subtropical Horticulture Research Station, the United States Department of Agriculture, Miami, FL; and the Departamento de Fitopatologia, Estación Experimental Tropical "Pichilingue", Instituto Naciónal Autonomo de Investigaciones Agropecurias, Quevedo, Ecuador. Much of the cacao seed used in project research at Gainesville was provided by Dr. Gustavo Enriques and Dr. Jorge Morea, Centro Agronómico Tropical de Investigación y Ensenañza (CATIE), Turrialba, Costa Rica.

5. METHODS AND RESULTS

The project was addressed in three directions (1) refinement of the automated system to reduce variation and improve reliability; (2) installation of the automated system at Pichilingue and its subsequent use in the cacao improvement activities; the study of heritability of resistance/tolerance to witches' broom; and (3) development of biotechnological methods to identify genetic markers indicative of resistance/tolerance to witches' broom.

THE AUTOMATED INOCULATION/INCUBATION SYSTEM

Inoculation of cacao with basidiospores of *Crinipellis perniciosa* presented serious problems relative to timing of plant development and availability of basidiospores. Storage of basisidiospores in any manner could be done for only a very short time, no longer than 7 days in water suspensions at 4 C (Frias 1987, Frias, et al 1993). The complications became more pressing relative to plant availability because seed was not always available during the 7 days, and for the Holliday Test, seedlings were to be 4 days of age. The agar block method allowed only for immediate use of inoculum on susceptible plant parts. The first indication that a solution to this impasse might be possible was provided by Frias (1987). Frias collected basidiospores in a 16% glycerol solution with 0.01% Tween 20 (a wetting agent) with the pH adjusted to 6.2 with concentrated NaOH. He also stored spores for up to 7 days at 4 C without loss of infectivity in the collecting solution. Suspensions of collected basidiospores were stored in the 16% glycerol collecting solution in liquid nitrogen. Dickstein, et al (1990) reported that viability and infectivity of stored basidiospores was very good after 18 months in liquid nitrogen.

To screen germplasm effectively and efficiently, the automated inoculation /incubation system was developed to apply basidiospores to cacao. A system for inoculating pine with basidiospores of the fusiform rust pathogen developed by the U.S. Forest Service, Asheville, North Carolina (Anderson, et al,1983), was the model for the system developed at the University of Florida, Gainesville, Florida.

All variables that influence infection of cacao by the witches' broom pathogen are controlled, and hundreds of individual seedlings, or vegetatively propagated plants, can be inoculated in a very few minutes. Inoculum concentration is controlled so that repeated applications of basidiospore can be made with the same concentration of viable basidiospores. The 16% glycerol collecting solution containing ungerminated basidiospores was diluted to >3% glycerol so that basidiospores could germinate (Frias, et al, 1995). This suspension of basidiospores was sprayed on to developing flushes that have at least one leaf 1.5 cm in length. Flushes at this growth stage are natural infection courts, and applications of basidiospores to these flushing shoots simulates the naturally occurring infection processes in the field. Inoculated plants are immediately placed in the incubation chamber where the relative humidity is 100% and the temperature is favorable for spore germination and infection (18-27 C). After 24 hours the inoculated plants are returned to the greenhouse, and symptom development is evaluated after 30 and 60 days.

A tremendous advance occurred with development of methods to store basidiospores of *Crinipellis perniciosa* in liquid nitrogen. Basidiocarps of *C. perniciosa* that develop in the laboratory or field produce basidiospores that are collected in the 16% glycerol collecting solution, and 2 ml of this suspension is placed in each cryoampule that is then placed in liquid nitrogen, or the collected basidiospores can be applied to cacao plants immediately. Basidiospores stored in liquid nitrogen have retained viability and infectivity for as long as 7 years. Although germination percentages decrease over time, the numbers of viable basidiospores applied to cacao can be repeated time after time by adjusting the total numbers of basidiospores in the inoculum. This breakthrough, coupled with the development of the inoculation/incubation system, provides for the first time the means by which germplasm and progeny of crosses (individual seedlings or families) can be evaluated effectively and efficiently for resistance/tolerance to witches' broom.

Results obtained during this project have demonstrated that basidiospores of *C. perniciosa* can be stored in liquid nitrogen for as long as 7 years (Dickstein and Purdy, 1996). Symptoms of witches' broom and percentages of disease that developed after inoculation with stored basidiospores were similar to those produced by freshly collected nonstored basidiospores (Table 1). With this technology the problem of inoculum availability has been solved. Also, it is possible to inoculate plant materials from the same or different sources over time with aliquants of the same original collection of basidiospores. This technology is in use at Pichilingue where two liquid nitrogen canisters, provided by this project, are in use to provide inoculum as needed.

Frias (1987) and Frias, et al (1993) presented data that supported the conclusion that plant materials must be in a certain stage of development or growth to be susceptible to the witches' broom pathogen. Regardless of the method of inoculation, cacao plant tissues that receive the inoculum must be in an unhardened stage of growth. Frequently, cacao seeds do not germinate precisely at the same time, and the resulting seedlings do not all grow at the same rate. The same can be observed with vegetatively propagated clones, the plants grow at different rates. As a result, an adequate number of plants might not be available for inoculation at any one time if they are not induced to develop similarly. To even out growth rate differences so that there are sufficient numbers of plants to run a well designed inoculation experiment, seedling and vegetatively propagated plants were decapitated by removing the terminal of developing plants that stimulated a flush and plants tended to attain a stage of development that was known to be susceptible to infection at a similar time.

Incubation of inoculated plants in the proper environment was essential for success, and the conditions within the incubation chamber that is part of the system provided the proper post-inoculation environment. A range of temperatures from 18-27 C and 100% relative humidity are essential to support basidiospore germination, penetration of the host, and establishment of the fungal pathogen within the host tissues (the infection site). During the tenure of this project, thousands of cacao seedlings were inoculated with basidiospores of *Crinipellis perniciosa* using the automated inoculation/incubation system. Disease that developed was recorded (Table 2) and individual seedlings that appeared to be free of symptoms, or had only minimal symptoms, were inoculated again, selected, and retained if they continued to display no or minimal symptoms after the second inoculation. Susceptible seedlings were autoclaved and discarded.

Plant Response Classification

A system was developed to classify plant responses to *Crinipellis perniciosa* that included all symptoms observed are named in the following categories:

<u>Responses</u>

1. No apparent symptoms
2. Broom from axillary or terminal bud
3. Roughened bark, enlarged lenticels
4. Hypocotyl swelling
5. Stem swelling
6. Swelling or broom at cotyledonary node
7. Pulvinus swelling

Broom Types

1. Diseased stem elongated with slight increase in stem diameter (< 1.5 the diameter of noninfected stems) growth from broom terminal, no or few buds on broom.
2. Stem enlarged (1.5-2.0 times diameter of noninfected stem), growth mostly at broom terminal, some axillary buds activated, a few small curled leaves on broom.
3. Enlarged stem (2.0 or more times the diameter of noninfected stem), growth from broom terminal and axillary buds of broom, curled leaves.
4. Necrotic broom
   a. <1/4 of broom necrotic
   b. 1/4 to 1/2 of broom necrotic
   c. >1/2 of broom necrotic

Data were recorded 30 and 60 days after inoculation. Longer periods (61-254 days) did not alter significantly the results observed at 60 days. Although all symptoms were recorded, broom types and frequencies of brooms were the most important indicators of disease, and were the symptoms used in selections of resistance/tolerance individual cacao seedlings that were designated GNV clones.

Thus, the problems of inoculum availability at the time plant materials are susceptible to infection by *Crinipellis perniciosa* were solved, and the mechanical components of the automated inoculation/incubation system that provided control of other variable that influence infection of cacao by basidiospore of *C. perniciosa* were in place.

During this project a total of 310 individual seedlings from 24 different cacao clones were selected for further evaluation following two inoculations of cacao seedlings with 75,000 basidiospores/ml. Individual seedlings that appeared to be free from symptoms or were mildly symptomatic were selected after the second inoculation and given a GNV (the airline designation for Gainesville, Florida) clonal number for future reference. More than 200 GNV clones were propagated vegetatively and the resulting plants were inoculated with basidiospores of *Crinipellis perniciosa* from Ecuador using the automated system. Plants from certain clones developed symptoms of witches' broom and the clone was rejected from further consideration.

The 34 apparently most resistant/tolerant GNV clones (Table 3), based on comparisons by Duncan's Multiple Range Test, were selected, and budwood of each of these clones was sent to the Cocoa Research Unit, Cocoa Genebank, The University of the West Indies, Trinidad, West Indies and will be included in that germplasm collection following a 2-year period in the cocoa quarantine facility in Barbados. The universal susceptible, Catongo, had a disease mean of 10.000. The clones GNV-313 and GNV-306 were the most resistant/tolerant GNV clones evaluated.

A collection of more than 150 mycelial cultures of *Crinipellis perniciosa,* assembled during the past 15 years, has been maintained for use in this project and other research. Also, basidiospores from various sources/locations have been stored in liquid nitrogen beginning in 1987, and about 1,000 2ml cryoampules of basisidiospores of *C. perniciosa* are presently in storage. These items will be transferred to Dr. D. B. Furteck, The American Cocoa Research

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THE AUTOMATED SYSTEM AT PICHILINGUE

The automated inoculation/incubation system was installed at Pichilingue and was used immediately in instructional experiments to familiarize individuals with its operation. During the course of the project more than 10,000 seedlings have been inoculated using the system. Classification of plant responses to infection by *Crinipellis perniciosa* based on plant responses recorded in Gainesville served as the base for classifications at Pichilingue, but responses not observed in Gainesville were added to the list of host plant responses; such as the extreme elongation of the stem above the cotyledonary node that almost always resulted in the death of the seedling.

An increased activity developed in Ecuador to restore the "naciónal" germplasm in the cacao industry of the country. Seedlings from either open pollinated or hand pollinated seed of 100 trees (clones or cultivars of the naciónal type) have been evaluated by the system, and are being characterized further with the intent of incorporating the most resistant/tolerant parents into the cacao breeding program. The distribution of the cacao clones (cultivars) in relation to percentages of infection by *Crinipellis perniciosa* are shown in Figure 1. About one third of the clones inoculated developed 25% or less disease, and clones that develop less than 50% disease will be evaluated again to determine if they might possess useful resistance/tolerance to the disease.

At least 40 seedlings from open pollinated seed from each of 56 trees (cultivars or clones) selected as "naciónal" types were inoculated, symptom development was classified, and results were analyzed by Sra. Julieta Rivera. These results will be included in her thesis for the Ingeneiro Agronomo degree. Ten of these clones selected as being the most resistant/tolerant (less than 25% disease in the seedling families) will be propagated vegetatively and the plants will be inoculated with the automated system to correlate the initial responses with responses of the vegetatively propagated plants.. The best clones will then be planted in the field with adequate replication to record field performances with respect to witches' broom and productivity. To date, selected individual seedlings from 12 other clones of the "naciónal" type have been planted in randomized field plots to observe disease responses and evaluate productivity.

Crosses of certain clones produced some rather interesting results relative to responses of families to *C. perniciosa*. For example, tree number 12 was crossed with trees 16, 75, 132, and 160; tree 44 was crossed with trees 15, 25, 60, 75, and 140; and tree 279 was crossed with tree 28. Seedlings from these crosses responded to witches' broom as shown in Figure 2 supporting a conclusion that tree 44 or trees 25, 75, and 140 possess what appeared to be very useful resistance/tolerance to the disease.

Interesting results developed following inoculation of four clones (SCA-12, EET-48, EET-62, and EET-103) in that all four clones appeared to be susceptible to witches' broom (Figure 3). Genes that influence resistance/tolerance appear to have been present in the progeny from the crosses SCA-12 x EET-48; and SCA-12 x EET-62. These results support a conclusion based on analyses of 20-year old data at Pichilingue that SCA-12 does, indeed, contribute resistance/tolerance genes in certain crosses Reinoculation of families of these crosses followed by selection and field evaluations of the most resistant/tolerant individuals may demonstrate that useful resistance/tolerance is present and can be used to improve plant materials for growers.

Heritability studies at Pichilingue to establish the heritability of resistance/tolerance to witches' broom suffered from the lack of adequate numbers of successful crosses because flowering of certain clones did not occur simultaneously. The experiment has been redesigned and additional attempts to make planned crosses is in progress so that the North Carolina II design can be completed with a cooperative effort that includes individuals in the witches' broom resistance/tolerance project and the newly assigned cacao breeder.

The automated system for inoculating cacao plants with basidiospores of the witches' broom pathogen was installed at Pichilingue as part of the project, and witches' broom resistant/tolerant "nacional" and other germplasm have been detected. The first ever field evaluation plots for plant materials selected via the automated system are established. The two principal objectives of the revitalized research at Pichilingue are: (1) To meet present needs of growers by providing vegetatively propagated witches' broom-resistant/tolerant clonal materials for immediate use in the field; and (2) To detect and develop through the breeding program genetically resistant/tolerant planting materials for grower use in the future. For the first time in many years a well trained and active cacao breeder, Ing. Luiz Duicela (MS in plant breeding/genetics in Mexico), who works well with all other personnel and programs at Pichilingue, has designed and begun an excellent program to breed cacao using "nacional" and other germplasm selected for various characteristics.

A total of 59 GNV clones have been transferred to Pichilingue for evaluation in the field. Hopefully, some of these clones may contribute useful genes for use in the cacao improvement program at Pichilingue or other locations. Mr. Soon Boo, a student at the University of Florida employed in this project, possesses excellent talent for propagating cacao vegetatively by budding. The American Cocoa Research Institute responded favorably to a request to support Mr. Boo to demonstrate his methods for personnel in Pichilingue by providing funds for him to be there for one week. The methods of propagation demonstrated were received with enthusiasm, and it is anticipated that propagation of cacao at Pichilingue will improve over the present level of success.

BIOTECHNOLOGICAL ASPECTS OF THE PROJECT

Leaves from cacao seedlings apparently resistance/tolerance to witches' broom were evaluated by isozyme analyses during the early years of this project. Results suggestive of a specific marker for resistance/tolerance to witches' broom was reported (Ronning, et al, 1991), but unfortunately this information was determined to be artifactual, and therefore invalid. Analyses for specific markers continued until the summer of 1992 and the arrival of hurricane Andrew that destroyed almost all facilities at the USDA Subtropical Horticulture Research Station, Miami, Florida that were devoted to cacao germplasm. Serious damage to laboratories also prevented any research for a extended time following the hurricane. As a result cacao research has been terminated at the station. Hurricane Andrew also destroyed the Cacao Quarantine facility and it has not been restored to a working condition. Thus, there is no longer a cacao quarantine program in the United States. In addition, all germplasm and the two Quonset-like plastic-screened houses in which the germplasm was growing were destroyed by hurricane Andrew.

The hurricane notwithstanding, the graduate research of C. M. Ronning continues at the University of Miami, Coral Gable, Florida, and is supported by The American Cocoa Research. Some of this research included preliminary analyses of bulked leaf samples from witches' broom resistant/tolerant plants with "naciónal" characteristics, and another bulked leaf sample from plants in the same plot that were apparently resistant/tolerant to Moniliophthora pod rot. Seedling trees that contributed leaves were in the first plot ever planted anywhere with cacao selected after inoculation with basidiospores of *Crinipellis perniciosa* using the automated inoculation/incubation system at Pichilingue. These leaf samples were screened with 26 RAPD primers, and result were that 10 bands unique to witches' broom resistant/tolerant sample, and 9 bands unique to the Moniliophthora resistant/tolerant sample were detected. Additional research is in progress to determine if there are markers indicative of specific resistance/tolerance among the bands detected.

Bulked leaf samples of 22 GNV clones that were apparently resistant/tolerant to witches' broom, and 19 GNV clones that were susceptible to witches' broom were sent to Miami for analysis to determine if any unique bands might be present that are indicative of resistance/tolerance to witches' broom (Table 4). Preliminary results with genomic DNA using random amplified polymorphoric DNA (RAPD) primers, indicate that there are unique bands presents from the resistant/tolerant leaf sample that are absent from the susceptible leaf sample. Additional screening is essential before conclusions can be drawn. The use of

RAPDs for genomic mapping and to study genetic diversity offers opportunities not previously available for genetic analyses of heterozygous woody perennials such as *Theobroma cacao*. An example of results obtained using RAPDs and cacao are presented by C. M. Ronning and R. J. Schnell (1995).

6. IMPACT, RELEVANCE, AND TECHNOLOGY TRANSFER

Results from this project are already contributing to the cacao programs in Ecuador. The transfer of the technology and installation of the physical components of the automated inoculation/incubation system at Pichilingue was the start of a new era for cacao research at this INIAP experiment station where the cacao research for the country is centered. Project activity began at the most opportune time possible in that the cacao industry of the country suffered declines in certain areas for several years. New plantings tended to overshadow losses in existing plantings, and time has passed during which the witches' broom disease has ravaged planting after planting of cacao. The favorable position of Ecuador cacao also declined because planting materials available for growers did not possess "naciónal" genes that impart the sought-after flavor of Ecuador cocoa beans. There is considerable effort in progress to find germplasm with "naciónal" characteristics and acceptable responses to witches' broom and other stresses that occur in the country. Candidate trees are evaluated for resistance/tolerance to witches' broom using the automated inoculation/incubation system installed at Pichilingue. This research could not have taken place prior to this project because the capability; to handle the pathogen and the plant material for high numbers of candidate plants, trees, or clones did not exist at Pichilingue or elsewhere in any cacao growing location in the world.

This project became the focus of activity at Pichilingue to find resistant/tolerant germplasm. INIAP and other agencies within Ecuador provided funds, and funds from external sources added significantly to improvements in the laboratory at Pichilingue. All of these improvements will add to the capability to achieve success by improving planting materials for grower use. There exists now a close working relationship between Dra. Carmen Suarez C. (Head Fitopatologia, Pichilingue) and Ing. Luis Duicela (newly assigned cacao breeder in the Cacao Program, Pichilingue). This new relationship has already advanced activity to the first ever field plots of selected individuals that passed through the automated inoculation/incubation system and showed resistance/tolerance to witches' broom. This present compatible relationship between two key individuals appears to assure proper attention to the goals of the station and country relative to cacao. Even if the capability to screen cacao germplasm had existed in prior years, progress would not have been made because of other personnel who would have been involved. The inoculation/incubation system, improved ways to handle the pathogen and plant materials, compatible individuals, and a country induced incentive -- all foretell success.

The technology and components of the automated inoculation/incubation system, a new 486 computer with abundant software including statistical programs, and miscellaneous laboratory supplies and materials are project residuals left at Pichilingue for witches' broom research in the Departamento de Fitopatologia at Pichilingue. With these residuals, research is now possible that could not be done before, and the expectation from this research, improved planting materials for growers, will become a reality.

7. PROJECT ACTIVITIES/OUTPUTS

Annually, except for one year, the project P. I.'s traveled to Pichilingue to evaluate project activity and plan research.

Sra. Julieta Rivera used project research to help with her thesis for the Ingeneiro Agronomo degree.

Publications planned:

Dickstein, E. R. and Purdy, L. H. Storage of basidiospores of *Crinipellis perniciosa* in liquid nitrogen. Plant Disease.

Ronning, C. M. and Schnell, R. J. RAPD's to detect genetic markers for resistance/tolerance to the witches' broom disease in *Theobroma cacao*. Hortscience.

Publications resulting from this project will carry the appropriate acknowledgment for support provided by US AID as stated in the guidelines for project reports.

8. PROJECT PRODUCTIVITY

Certain original objectives could not be met because of technical and logistical problems. For example, it was not possible to correlate field performance of clones with their respective performances under controlled inoculation conditions, except at Pichilingue. This research required vegetative reproduction of clones that failed at Pichilingue, Gainesville, and Miami. Variation in pod and vegetative susceptibility in the field has been done over the years at Pichilingue, but there was no attempt to retrieve that archival data, and there was no apparent reason to repeat former experiments. However, with the new thrust at cacao naciónal such comparisons are in progress. Likewise heritability estimates for witches' broom resistance are being studied in the field at this time in Pichilingue. Because of several failures to propagate cacao vegetatively in sufficient numbers to be meaningful, it was not possible to evaluate the witches' broom resistance/tolerance of selected plants in production plots in Puerto Rico.

9. FUTURE WORK

Within the text of this report there are several statement that relate to future work at Pichilingue. There is ample evidence that personnel are vitally interested in "naciónal" germplasm with resistance/tolerance to witches' broom, and they are seeking the best trees with the combined efforts of many individuals. The equipment, supplies, and materials left at Pichilingue combined with the technology to use them will yield results that will improve the present status of growers and the cacao industry of Ecuador. Personnel and other changes within INIAP and specifically Pichilingue make the future of cacao improvement very bright. A vigorous, established, and youthful cacao breeder is now part of the cacao program, and is an individual who will produce whereas such desire seemed to be absent in the past. It appears that the financial problems faced by personnel at Pichilingue in the past might have decreased in intensity, but extra mural support will still be needed to move forward with the speed that enthusiasm can generate.

10. LITERATURE CITED

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TABLE 1. Germination percentages and percentages of infection by basidiospores of <u>Crinipellis perniciosa</u> from three sources stored in liquid nitrogen for four years.

Source of spores	Date Stored	Germ. Date	percent Germ	# months storage	Inoculum Conc.	Percent Infect.(# infectected/#inoculated) Catongo	EET 400	SCA 12
Colombia	6-29-90		80.0	0				
		6-22-94	56.1	48	75,000	100 (15/15)	100 (15/15)	100 (6/6)
		7-26-94	55.6	49	75,000	100 (15/15)	93.3 (14/15)	80.0 (12/15)
Bahia	7-01-90		77.0	0				
		6-22-94	67.9	47.75	75,000	86.6 (13/15)	73.3 (11/15)	33.3 (2/6)
		7-26-94	66.7	48.75	75,000	100 (15/15)	86.6 (13/15)	53.3 (8/15)
Trinidad	7-01-90		30.0	0				
		6-22-94	20.5	47.75	75,000	80.0 (12/15)	53.3 (8/15)	83.3 (5/6)
		7-26-94	23.0	48.75	75,000	80.0 (12/15)	46.6 (7/15)	60.0 (9/15)

TABLE 2. Illustration of data recorded for each inoculated seedling or vegetatively propagated cacao plant.

Cacao Variety	Seed Source	Previous inoc. dates	Total # of plants inoc.	# of plants died	Broom rating after 30 days # and (%) 3	2	1	Astmpt. plants # and (%)	Broom rating after 60 days # and (%) 3	2	1	Asympt. plants # and (%)
ACT 2-5	Miami	12-03-91	5	0	2 (40)	0 (0)	2 (40)	1 (20)	4 (80)	0 (0)	0 (0)	1 (20)
		12-06-91	9	0	3 (33.3)	0 (0)	2 (22.2)	4 (44.4)	5 (55.6)	1 (11.1)	0 (0)	3 (33.3)
Catongo	CATIE	1-28-92	9	0	5 (55.6)	0 (0)	0 (0)	4 (44.4)	5 (55.6)	0 (0)	1 (11.1)	3 (33.3)
EEG 64	Miami	11-08-91	8	0	1 (12.5)	1 (12.5)	0 (0)	6 (75)	2 (25)	0 (0)	1 (12.5)	5 (62.5)
PA 276	Miami	12-06-91	5	0 (1)	2 (40)	1 (20)	0 (0)	2 (40)	3 (60)	0 (0)	0 (0)	1 (20)
SCA 12	CATIE	11-05-91	3	0	0 (0)	0 (0)	2 (66.7)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0)	1 (33.3)
		1-28-92	24	0	9 (37.5)	3 (12.5)	3 (12.5)	9 (37.5)	14 (58.3)	3 (12.5)	0 (0)	7 (29.2)

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Table 3. GNV clones listed in order of increasing resistance/tolerance to *Crinipellis perniciosa* for inclusion in the Cocoa Genebank, Cocoa Research Unit, The University of the West Indies, Trinidad, West Indies.

Clone No.	Maternal Parent	Source	Disease mean	Duncan's group
189	EET 387	PICHILINGUE	4.750	ABCDEFG
268	IMC 67	USDA_MIAMI	4.500	ABCDEFG
12	UF 613	USDA-MIAMI	4.300	ABCDEFG
152	CATONGO	CATIE	4.250	ABCDEFG
266	ICS 95	USDA-MIAMI	4.200	ABCDEFG
141	SCA 6	CATIE	4.150	ABCDEFG
327	P 43	USDA-MIAMI	4.000	ABCDEF
180	EET 61	PICHILINGUE	4.000	ABCDEFG
19	UF 613	USDA-MIAMI	4.000	ABCDEFG
97	SCA 6	CATIE	4.000	ABCDEFG
86	PA 120	USDA-MIAMI	3.700	ABCDEFG
30	SCA 12	CATIE	3.688	ABCDEFG
263	EET 387	USDA-MIAMI	3.600	ABCDEFG
105	EET 400	CATIE	3.250	ABCDEFG
331	SCA 9	USDA-MIAMI	3.250	ABCDEFG
314	113 R	USDA-MIAMI	3.250	ABCDEFG
286	SCA 12	CATIE	3.000	ABCDEFG
258	EET 64	USDA-MIAMI	2.875	ABCDEFG
38	SCA 12	CATIE	2.833	ABCDEFG
287	SCA 12	CATIE	2.708	ABCDEFG
63	SCA 12	USDA-MIAMI	2.500	ABCDEFG
47	SCA 12	USDA-MIAMI	2.600	ABCDEFG
22	EET 400	CATIE	2.500	ABCDEFG
293	SCA 12	CATIE	2.500	ABCDEFG
225	SCA 12	CATIE	2.467	ABCDEFG
42	ICS 95	USDA-MIAMI	2.375	BCDEFG
120	SCA 12	CATIE	2.350	BCDEFG
31	SCA 12	CATIE	2.167	CDEFG
295	SCA 12	USDA-MIAMI	2.111	CDEFG
113	SCA 12	CATIE	2.000	DEFG
111	SCA 12	CATIE	1.875	EFG
33	SCA 12	CATIE	1.500	FG
313	75R	USDA-MIAMI	1.313	G
306	UNK 59	PICHILINGUE	1.000	G

Table 4. Identities, female parent, and seed sources of GNV clones for analysis by RAPD screening to detect resistance/tolerance markers to witches' broom.

GNV no.	Female parent	Source	GNV no.	Female parent	Source
		RESISTANT/TOLERANT			
24	SCA-12	CATIE	141	SCA-12	CATIE
31	SCA-12	CATIE	163	CATONGO	CATIE
32	SCA-12	CATIE	258	EET-64	PICHILINGUE
38	SCA-12	CATIE	280	SCA-12	CATIE
42	ICS-95	USDA-MIAMI	295	SCA-12	USDA-MIAMI
53	SCA-12	USDA-MIAMI	313	75 R	USDA-MIAMI
86	PA-120	USDA-MIAMI	314	113 R	USDA-MIAMI
97	SCA-6	CATIE	331	SCA-9	USDA-MIAMI
113	SCA-12	CATIE	327	P-43	USDA-MIAMI
120	SCA-12	CATIE	334	SCA-12	CATIE
		SUSCEPTIBLE			
23	EET-400	CATIE	259	EET-64	PICHILINGUE
72	EET-400	CATIE	264	EET-387	USDA-MIAMI
76	P-43	USDA-MIAMI	265	EET-387	USDA-MIAMI
80	SPA-9	USDA-MIAMI	279	SCA-12	USDA-MIAMI
96	ICS-1	USDA-MIAMI	300	SCA-12	USDA-MIAMI
109	SCA-12	USDA-MIAMI	301	SPA-9	USDA-MIAMI
149	SCA-12	CATIE	326	M-109	USDA-MIAMI
164	UF-613	USDA-MIAMI	330	PA-276	USDA-MIAMI
203	IMC-67	USDA-MIAMI	337	UNK #2	USDA-MIAMI
220	SCA-6	PICHILINGUE			

Figure 1. Distribution of cultivars of cacao in relation to percentages of witches' broom infection..

Figure 2. Behavior of families of various crosses inoculated with basidiospores of *Crinipellis perniciosa* using the automated inoculation incubation system.

Figure 3. A. Percentages of infection by *Crinipellis perniciosa* of seedlings from four clones.

B. Percentages of infection by *Crinipellis perniciosa* of families from three crosses: SCA-12 x EET-48; SCA-12 x EET-62; SCA-12 x EET-103.

FIGURA No. 1

Distribucion de cultivares de cacao en relacion a porcentaje de infeccion

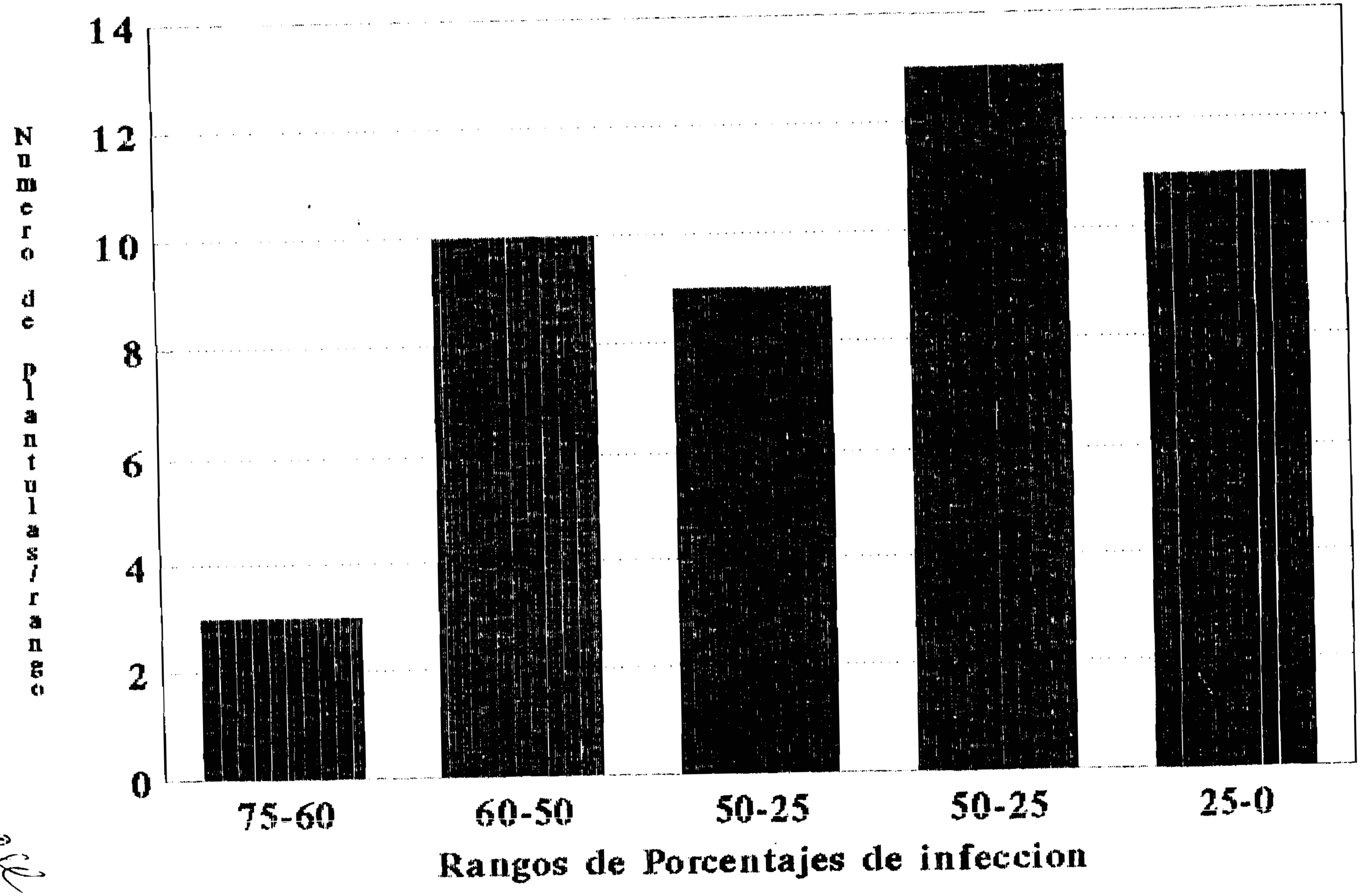

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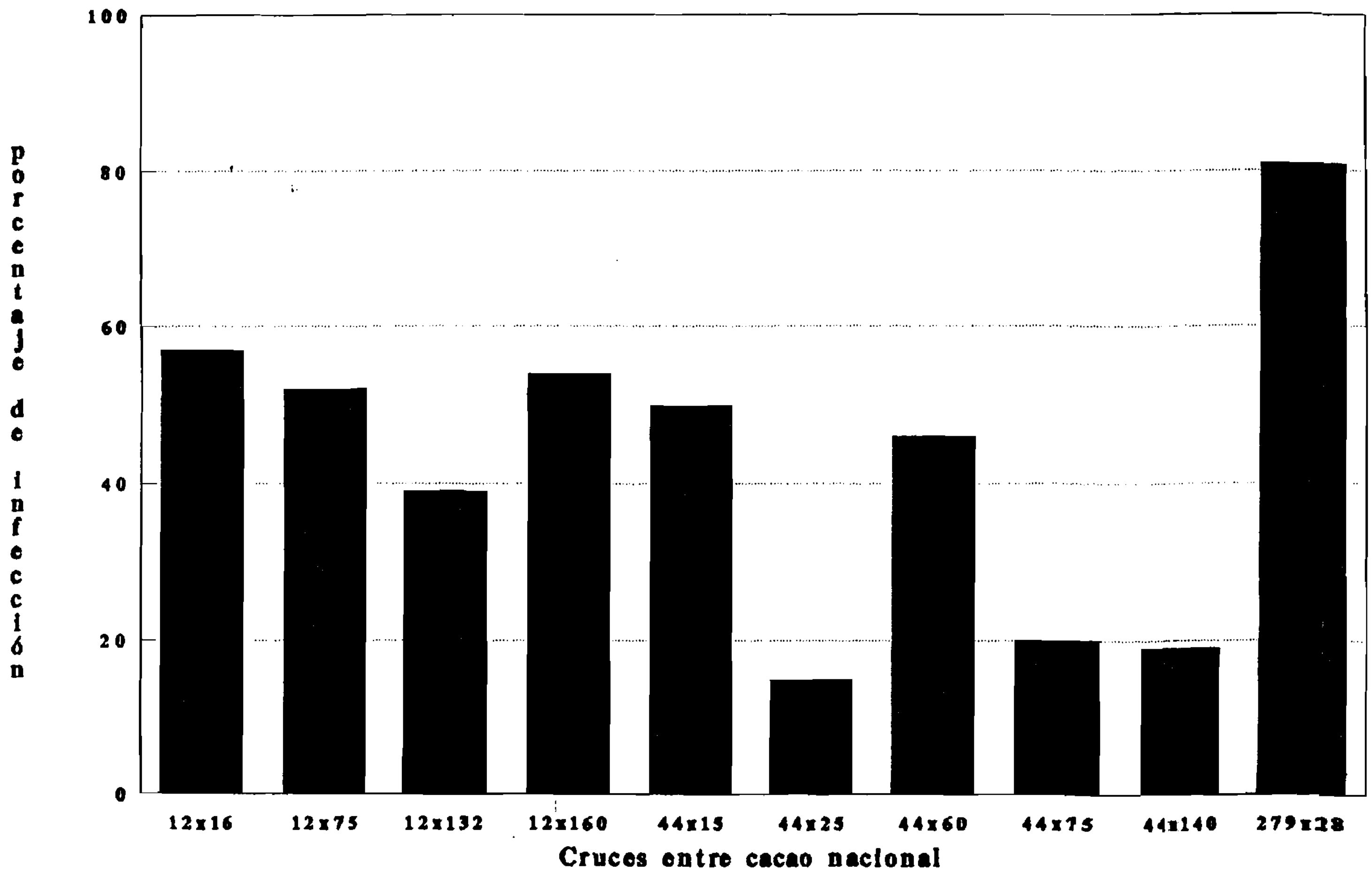
FIGURA No. 2

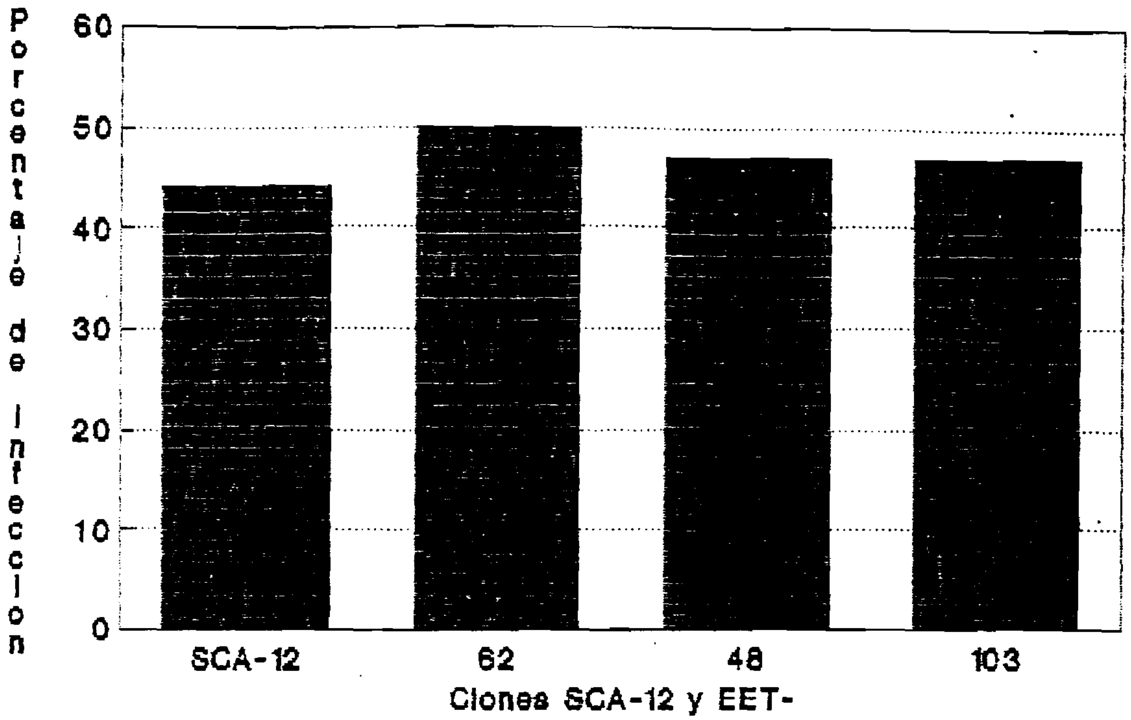
Comportamiento de varios cruces inoculados con C. perniciosa.



Números corresponden a No. de cultivar de 'La Buseta'

FIGURA No. 3

Comportamiento de plantulas de semilla clonal, frente a *C. perniciosa*



Comportamiento de plantulas inoculadas con *C. perniciosa*